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(54) Title: NOVEL METHOD OF LARGE SCALE PLASMID PURIFICATION

(57) Abstract

A method of purifying pharmaceutical grade plasmid DNA from host cells is disclosed. The method involves a single, "mixed mode" anion exchange step which employs a stringent ethanol wash to remove endotoxins and other impurities from more hydrophilic plasmid DNA.

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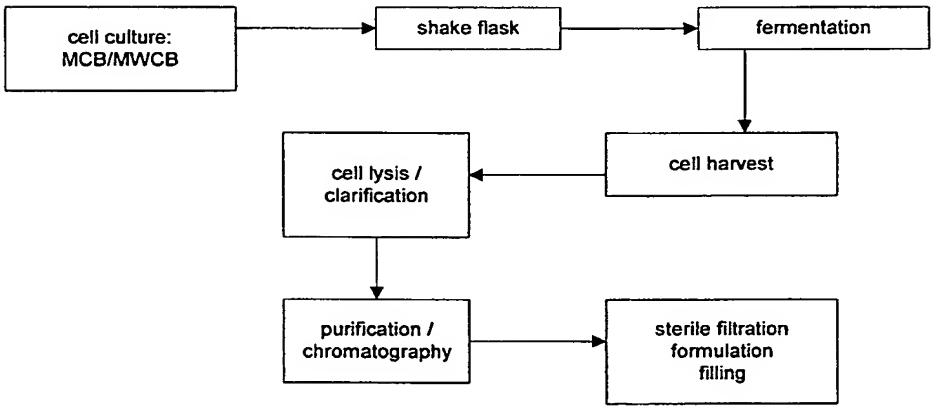
NOVEL METHOD OF LARGE SCALE PLASMID PURIFICATION

Background of The Invention

With the increasing value of recombinant DNA in therapeutic applications, the need for methods for obtaining sufficient amounts of highly pure plasmid DNA from cell cultures has grown.

Typically, the manufacture of large scale plasmid DNA involves the following steps.



Within this general scheme, chromatographic purification of DNA presents perhaps the biggest hurdle based on the physical characteristics of the biomolecule as well as the intrinsic impurities derived from the host cell, for example, *E. coli.* The chief impurities which hinder the purification of plasmid DNA are the large amounts of polymers of similar structure (chromosomal DNA and RNA) and high levels of endotoxin.

Plasmid DNA is a highly anionic polymer which is sensitive to shear and to degradation by nucleases. Plasmids are as large or larger than the pores of almost all chromatographic media. Several chromatographic procedures for the purification of biologically active plasmid DNA (without the use of CsCl - ethidium bromide ultracentrifugation) have been developed, at least at laboratory scale, including gel filtration chromatography, hydroxyapatite chromatography, acridine yellow affinity

chromatography, anion exchange chromatography, reversed phase chromatography, silica membrane binding and binding to glass powder. However, many of these methods are not well suited to the purification of large quantities of DNA.

In choosing a method of purification for large scale production of plasmid DNA, the most important consideration is the fact that the biomolecule is a highly anionic polymer which is sensitive to shear and to degradation by nucleases. Any large scale manufacturing process must address each of these concerns. Currently, the most successful methods of extraction and purification involve large scale alkaline lysis in SDS (Horn et al. (1995) *Human Gene Therapy* 6:565). This step efficiently removes chromosomal DNA, nuclease enzymes and other contaminants. Therefore, cell lysis conditions must be carefully optimized and low shear mixing must be used during this step. Plasmid extracts are primarily contaminated with low molecular weight cell components, process chemicals and RNA. These contaminants and trace host protein contamination generally are then removed by a combination of ultrafiltration, selective precipitation, anion exchange chromatography and, if necessary, a final polishing step. Accordingly, typical manufacturing protocols for large scale plasmid DNA involve several purification steps.

For example, one of the major drawbacks of using anion exchange chromatography in conventional purification methods as the sole high resolution purification step is that a portion of endotoxin and pyrogen contaminants will co-purify with the plasmid. Given the limitations of currently available commercial matrices and the often similar structure / charge profile of biomolecule species passing over the column, anion exchange has historically been used as the primary capture and initial purification step. However, a second polishing step which is orthogonal to the principles of anion exchange has always been necessary to lower the residual endotoxin level. Depending on the chromatographic parameters employed, residual RNA levels can also be unsatisfactory using this methodology.

In addition to the drawbacks discussed above, conventional plasmid purification protocols generally employ animal derived enzymes such as lysozyme, proteinase K and RNase. These enzymes are typically incorporated into laboratory "mini prep" kits and,

while they enhance the resolution and separation of the "mini prep" anion exchange matrices, they are totally unsuitable for use within a regulatory compliant cGMP production process.

Improved methods for obtaining large scale plasmid DNA in a highly pure form suitable for therapeutic use would be highly beneficial.

Summary of the Invention

The present invention provides a fully scaleable method for isolating plasmid DNA to a level of purity which meets regulatory standards required for the manufacture of recombinant protein pharmaceuticals. The method provides the advantage of streamlining the purification process so that a single anion exchange step is capable of producing pharmaceutical grade plasmid DNA. The method provides the further advantage of not requiring the use of enzymes or other agents capable of damaging the plasmid DNA, such as lysozyme, RNase, Proteinase K, phenol, chloroform, and ethidium bromide.

In one embodiment, the method of the invention calls for lysing host cells and obtaining a lysate; applying the lysate to an anion exchange column; washing the column with a solution comprising a sufficient amount of ethanol to substantially remove endotoxin bound to the column, without substantially removing plasmid DNA bound to the column; eluting plasmid DNA bound to the column; and collecting chromatographic fractions of the elute. The elute can then be assayed for purity using, for example, standard protein, DNA and/or endotoxin assays. Plasmid DNA present in the elute can be assayed for retained function using, for example, expression assays.

Further steps can optionally be performed in the method of the invention. In one embodiment, the lysate is clarified by filtering to remove large cellular debris prior to applying the lysate to the anion exchange resin. The lysate can also be concentrated to remove RNA precipitate. In a preferred embodiment, the lysate is mixed with a non-ionic detergent, such as 2% triton X-114, prior to applying the lysate to the column. Preferably, the detergent is one which flows through the anion exchange column and does not interfere with plasmid DNA binding to the resin. By performing this initial

phase separation step, hydrophobic contaminants (e.g., endotoxins and other protein contaminants), which are retained in the detergent phase of the mixture, can be separated from plasmid DNA which remain in the solution phase of the mixture. The detergent phase can be removed prior to applying the mixture to the anion exchange column, or the mixture can be directly applied to the column where the detergent and impurities contained therein will be separated from the plasmid DNA using the stringent ethanol wash.

In a preferred embodiment, the invention employs an ethanol wash containing between 25% to 95% ethanol, more preferably at least about 40% ethanol. The wash also preferably includes a lesser percentage of a neutralizing agent, such as acetic acid ranging from 5-10%.

In addition to the detergent phase separation step and the stringent (i.e., 25-95%) ethanol wash a second column step (e.g., anion exchange, affinity, size exclusion) can be employed in the method of the invention to ensure rigorous product control.

Brief Description of the Figures

Figure 1 is a schematic flow chart showing various purification steps of the invention.

Figure 2 is a chromatogram showing plasmid purification and endotoxin removal by TMAE standard chromatography.

Figure 3 is a chromatogram showing plasmid purification and endotoxin removal by 2% triton X-114 phase partitioning, followed by TMAE chromatography.

Figure 4 is a chromatogram showing plasmid purification and endotoxin removal by TMAE chromatography using a 40% ethanol + 5% acetic acid wash step.

Figure 5 is a chromatogram showing plasmid purification and endotoxin removal by 2% triton X-114 phase partitioning, followed by TMAE chromatography using a 40% ethanol + 5% acetic acid wash step.

Figure 6 1% shows PAGE (1% agarose gel) analysis of plasmid purification using TMAE standard chromatography.

Figure 7 shows PAGE (1% agarose gel) analysis of plasmid purification using 2% triton X-114 phase partitioning, followed by TMAE chromatography.

Figure 8 shows PAGE (1% agarose gel) analysis of plasmid purification using 2% triton X-114 phase partitioning, followed by TMAE chromatography using a 40% ethanol + 5% acetic acid wash step.

Figure 9 shows graphic and tabular results from transfection expression assays using plasmid DNA encoding α-interferon, purified by the method of the invention.

Detailed Description of the Invention

The present invention provides a novel "mixed" mode plasmid purification method which relies on differences in both charge and hydrophilicity between plasmid DNA and cellular impurities, such as endotoxins and other protein contaminants, to isolate plasmid DNA. These differences are exploited, in a single chromatography step, to produce plasmid DNA to a level of purity which meets regulatory standards required for the manufacture of recombinant protein pharmaceuticals (e.g., < 0.1 EU/μg DNA). The chromatography step employs both anion exchange and charge separation strategies, using a stringent ethanol wash, to separate endotoxins from plasmid DNA.

As in all plasmid purification protocols, the method of the invention initially calls for lysis and plasmid extraction from host cells containing the desired (e.g., recombinant) plasmid. This can be done, for example, by adding cells to alkaline buffers, such as 0.2 NaOH/1.5% SDS, as is well known in the art. Preferably the lysis

buffer does not contain animal derived, or other enzymes, such as lysozyme. Alkaline buffer extraction is used precipitate non-plasmid impurities, such as chromosomal DNA, RNA and cellular proteins.

Following lysis, the lysate is collected and preferably clarified, for example, by ultrafiltration through a suitable filter having a pore diameter of about 0.1 to 100 microns. Following clarification, the lysate is preferably concentrated using techniques known in the art, such as dialysis, diafiltration or centrifugation, and the resulting pellet resuspended in buffer. To precipitate residual RNA, the lysate also can be incubated (e.g., overnight) in ammonium acetate solution (e.g., at 4°C).

In a preferred embodiment of the invention, the lysate containing the plasmid DNA is combined with a non-ionic detergent, such as Triton X-114 (2%). Triton X-114 has the property of causing a separation between the detergent and the aqueous phases at temperatures above 20°C, its cloud point. Hydrophilic bio-molecules remain in the aqueous phase while integral membrane proteins and other hydrophobic species may be partitioned in the detergent phase. Accordingly, Triton X-114 can be added to the plasmid DNA supernatant (recovered after the ammonium acetate precipitation) for a final concentration of Triton X-114 of approximately 1-5%, preferably 2%. After stirring and incubation, the visible Triton X-114 phase (containing hydrophobic contaminants) can be removed using, for example, a pipette. However, this is not required since the detergent and contaminants therein can be removed by the chromatography step of the invention alone.

In particular, following preparation of cell lysate and, optionally, detergent phase partitioning as described above, the lysate is applied to a suitable anion exchange column.

Differences in ionic charge, molecular size, and/or other characteristics are exploited to bring about purification of the desired plasmid DNA species. While anion exchange is the preferred form of chromatography for use in the invention, other forms of chromatography which can be employed include, for example, size exclusion chromatography, reversed phase chromatography, hydrophobicity interaction chromatography, affinity chromatography, or any combination of these to bring about

the final purification of plasmid DNA. These chromatography techniques are well known in the art and can be practiced as described, for example, in US Patent No. 5,561,064, the contents of which are incorporated by reference herein.

In the present invention, anion exchange chromatography is performed to separate plasmid DNA molecules from contaminating molecules based on molecular ionic charge or isoelectric point (pI) at a given pH. Ion exchange columns may be packed with positively charged beads (for anion exchangers) that make up the support matrix. The charge density and pI of the molecules will determine the ionic capacity of the support matrix that is suitable for separating the molecules. Ion exchange operations may be run using two different mobile phases or buffers (i.e., under gradient conditions). The starting buffer may be a low salt or ionic concentration buffer. The eluting buffer may have a significantly higher ionic concentration than the starting buffer. The operating pH can be determined by sample solubility and support matrix stability. For example, an ion exchanger may be run at a pH of about 6-11 and a linear gradient developed between about 0.3 and 1.0M NaCl. Water miscible organic solvents (for example, acetonitrile) may be used to decrease retention time, but it is preferred that the use of organic solvents be avoided so that the storage of organic chemical waste is precluded.

In one embodiment, the invention employs a trimethylaminoethyl (TMAE) fractogel anion exchange resin. The resin is packed into a standard, preferably large-scale column, such as a Pharmacia XK 50 column, at a suitable bed height (e.g., approximately 20.5 cm) and a suitable total column volume (e.g., approximately 400 ml). The column is then run on a suitable preparative HPLC at a linear flow rate of e.g., 150 cm/hr. Chromatographic profiles are then monitored at two different wavelengths, for example, 260 nm and 280 nm, and peak fractions collected based on their real time chromatographic profiles.

Following packing, the column is equilibrated and the sample containing the plasmid DNA is loaded onto the column. Typically, the first wash contains relatively high salt (e.g., 0.68 M NaCl) and results in selective binding of components contained in the sample to the column, and removal of most of the detergent. This optional wash is

then followed by a stringent alcohol (e.g., ethanol) wash which removes both endotoxins bound to the column, and any residual detergent (e.g., Triton X-114), without removing plasmid DNA bound to the column. The term "stringent", as used herein, means a concentration of organic solvent (e.g., alcohol) of between 25% and 90%, more preferably approximately 40%. While ethanol is the preferred alcohol for use in the stringent wash, other alcohols know in the art, such as methanol, also can be used. The stringent wash also preferably contains a smaller percentage of a neutralizing acid, such as acetic acid. When using ethanol at the preferred concentration of about 5%, acetic acid is preferably present in the wash at a concentration of about 40%. The column is then finally eluted to capture the remaining plasmid DNA bound to the column.

Accordingly, a preferred chromatographic protocol for use in the present invention can be summarized as follows:

Column Equilibration:	50 mM Tris, pH 7.5, 500 mM NaCl
Sample Load:	Plasmid in 50 mM Tris, pH 7.5, 500 mM NaCl
Column Wash:	50 mN Tris, pH 7.5, 0.68 M NaCl
Stringent Wash:	**40 % Ethanol + 5 % Acetic Acid**
Column Elution:	50 mM Tris, pH 7.5, 1.2 M NaCl
Column Cleaning:	0.2 N NaOH + 1 M NaCl
Column Regeneration:	0.1 N HCl + 1 M NaCl

By increasing the amount of organic solvent in the stringent wash step, the plasmid purification method of the invention shifts the chromatographic separation from a purely ionic mode to that of a "mixed" mode. Organic solvent concentrations of 20 % or less do not aid in the removal of endotoxin, nor do they enhance the normal chromatographic resolution achieved with Fractogel TMAE. This is typically the concentration of organic solvent employed within silica based, plasmid "mini prep" kits. However, by using a stringent 40 % ethanol and 5 % acetic acid wash step (following the standard NaCl wash step) the physical differences between endotoxin and plasmid DNA are exploited to separate the two. The inclusion of 5 % acetic acid adheres to the

convention of performing hydrophilic chromatography at a low pH. The lower pH of the wash step can also aid in residual RNA removal.

A second column step also may be incorporated within the production process to add a degree of ruggedness and to ensure rigorous product control.

Following collection of elute, samples can be tested for plasmid DNA purity, quality and quantity using a number of assays known in the art. For example, to assay for endotoxin levels, a Limulus Amboycte Lysate Assay (LAL) kinetic assay can be performed on individual plasmid fractions according to manufacturer protocols. To analyze purified plasmid DNA for total size, and for the presence of residual genomic DNA and RNA, standard polyacrylamide gel (e.g., 1%) electrophoresis (PAGE) can be used. To analyze plasmid DNA concentration, individual fractions can be analyzed by optical density (OD) at a wavelength of 260 nm using and extinction coefficient of 50 μg / ml. To analyze samples for residual protein content, a Micro Bicinchoninic Acid (BCA) Assay kit (e.g., Pierce Micro BCA) can be used according to manufacturer protocols.

In addition and, typically, as a final step, purified plasmid DNA samples can be assayed for activity using expression or other functional assays. Transfection efficiency of plasmid DNA can be used, for example, using reporter genes or genes encoding protein which is then quantified in standard immunoassays.

The invention shall be further illustrated in the following examples which are not intended to be limiting.

EXAMPLES

EXPERIMENTAL MATERIALS

"TEG" Buffer (25 mM Tris / 10mM EDTA / 50mM Glucose, pH 7.5)

0.2 NaOH + 1.5% SDS Buffer

3 M Potassium Acetate + 5 M Acetic Acid Buffer, pH 5.6 (KAc / AA)

"TE" Buffer (50mM Tris +10mM EDTA, pH 7.5)

Ammonium Acetate (NH_4Ac)

Triton X-114

50mM Tris buffer pH 7.5

50mM Tris buffer +1.2 M NaCl, pH 7.5

0.2 N NaOH + 1M NaCl

0.1 N HCl + 1 M NaCl

40% Ethanol + 5% Acetic Acid

E.M. Science Fractogel TMAE Resin (45-90μM)

Pharmacia XK 50 cm x 20.5 cm column

BioCAD 250 Preparative HPCL

Amicon SP 20 Ultrafiltration Unit

Amicon 100,000 molecular weight (mw) cutoff Ultrafiltration cartridge (S10Y100 spiral membrane)

Braun Biostat C Fermentor (30 L working volume)

Fermentation Media Components: (see also Figure 1)

Seed Media

K_2HPO_4 5.5 g/L

$(NH_4)_2SO_4$ 3.75 g/L

$(NH_4)_2HPO_4$ 5.5 g/L

$MgSO_4 \cdot 7H_20$ 2.0 g/L

Yeast Extract 20 g/L

Trace Metal Solution 5 ml/L

Trace Metal Solution

$FeCl_3 \cdot 6H_2O$, 6.46 g/L

$CaCl_2 \cdot 2H_2O$, 6.0 g/L

$ZnSo_4 \cdot 7H_2O$, 2.81 g/L

$MnCl_2 \cdot 4H_2O$, 1.2 g/L

$CuSO_4 \cdot 5H_2O$, 0.3 g/l

H_3BO_3, 0.12 g/L

$(HN_4)_6Mo_7O_{2/1} \cdot 4H_2O$, 0.12 g/L

$CoCl_2 \cdot 6H_2O$, 0.12 g/L

HCl, 37 ml

Glucose 4 g/L

Fermentation Media

K_2HPO_4, 8.25 g/l

$(NH_4)_2SO_4$, 5.625 g/L

$(NH_4)_2HPO_4$, 8.25 g/L

$MgSO_4 \cdot 7H_2$, 0.5 g/L

Yeast Extract, 10 g/L

$FeCl_3 \cdot 6H_2O$, 0.2025 g/L

$CaCl_2 \cdot 2H_2O$, 0.1875 g/L

$ZnSo_4 \cdot 7H_2O$, 0.877 g/L

$MnCl_2 \cdot 4H_2O$, 0.0375 g/L

$CuSO_4 \cdot 5H_2O$, 0.0094 g/L

H_3BO_3, 0.00375 g/L

$(HN_4)_6Mo_7O_{2/1} \cdot 4H_2O$, 0.00375 g/L

$CoCl_2 \cdot 6H_2O$, 0.00375 g/L

Antifoam, 0.05 ml/L

Thiamine, 0.31 g/L

L-leucine, 0.31 g/L

Feed Medium

Glucose, 640 g/L

Yeast Extract, 100 g/L

$MgSO_4 \cdot 7H_20$, 12 g/L

Beckman J2-MI Centrifuge

Komposite 6 x 1 L Rotor

Materials and Equipment necessary to perform 3-QC-0048 (Kinetic LAL Assay)

Beckman DU-70 Spectrophotometer

Materials and Equipment necessary to perform Pierce Micro BCA assay

Materials and Equipment necessary to perform the Geno Technology™

1 % agarose gel assay

Materials and Equipment necessary to perform the CytoScreen™ Immunoassay

Materials and Equipment necessary to perform the Qiagen™ Maxi Prep Kit

***All buffer reagents used were J.T Baker Ultrapure Reagent or equivalent. All buffers were formulated in endotoxin free, nanopure water.

EXPERIMENTAL METHODS

EXPRESSION PLASMID AND BACTERIAL STRAIN

The plasmid pSBKIFNα contains the entire coding region plus 390bp of 3' untranslated flanking sequence (3'-UTR) of the human interferon α2b(IFN-α2b) gene. The IFN-α2b gene is under the transcriptional control of the human thyroxine-binding globulin promoter and α1-microglobulin/bikunin precursor protein enhancer elements. These are arranged on a pUC19-based vector (pBKKCMV) which contains the colE1 origin of replication and the aminoglycoside acetyltransferase gene driven by the beta-lactamase promoter. The IFN-α2b gene originated from plasmid pALCA1SIFN, which was obtained from the ATCC (Rockville, MD). Once the IFN-α2b gene from pALCA1SIFN was subcloned into pBKCMV, the 5' end of the gene was modified to match the endogenous human leader sequence of IFN-α2b, preceded by the Kozac consensus sequence (*J.Mol. Biol.* 235: 95-110, 1994). The plasmid also carries the kanamycin resistance gene. The plasmid DNA was propagated in the *E. coli* strain DH10B.

PRODUCTION OF BACTERIAL CULTURE

A 20% glycerol stock culture was used to inoculate four side-baffled flasks containing the seed medium with the appropriate concentration of kanamycin. The growth conditions were set a 32°C and 300rpm on a platform shaker. The seeds were

grown to an optimal density for transfer to the fermenter as determined by optical density (OD) at 600nm.

The Fed-Batch Fermentations were run in a B.Braun Biostat C-30 Fermenter with a starting working volume of 24L. The basal medium was inoculated with a 10% inoculum and fed using a mathematical model. The feed rate was adjusted as growth slowed to maintain less than 1.0 g/L glucose concentration in the fermenter.

Feed Equation:

$F = FO^* \exp(\mu(t-t_0))$, where:

$F0 = (\mu^*X)/(Y^*S)$

F, F0 in ml/hr (rate)

μ in h-1 (specific growth rate)

t in hours of fermentation

X in g total initial biomass

Y in g/g (yield of cell dry wt per gram of glucose)

S in g/ml (concentration of feed)

During fermentation, the aeration was maintained at 1vvm (vol. of air per vol. of liquid per minute) and dissolved oxygen was controlled at 20% by agitation. The pH was maintained at 7.0 with 28-30% NH_4OH. Temperature was held constant at 32°C in order to maintain a maximum growth rate (μ) of 0.31 Upon inoculation, the "feed" medium (see Appendix 1) was fed into the fermenter according to the feed rate profile. The duration of the fermentations were ~22 hrs or until late log phase was reached. Cells were then harvested by centrifugation and frozen at -20°C.

CELL LYSIS AND PLASMID EXTRACTION

To 2000g of cells (wet weight), 9.0 L of TEG buffer was added until the cells were completely thawed and dispersed in solution. To this cell suspension, 21.0 L of 0.2 N NaOH +1.5 % SDS buffer was added and incubated at room temperature with thorough mixing for twenty minutes. After twenty minutes, 16.5 L of KAc / AA buffer

was added and this mixture was incubated at 4°C (ice bath) with constant mixing for thirty minutes. Large cellular debris was removed by filtration through a minimum of 2 layers of Miracloth™. The partially clarified lysate was then filtered through a 3.0 μM Sartofine filter before application to the Amicon SP 20 ultrafiltration unit. The lysate was concentrated to a volume of approximately 5L using an Amicon 100,000 MW cutoff spiral wound membrane by maintaining an inlet pressure of 1.5 bar and an outlet pressure of 0.8 bar. Temperature was kept at <20°C by using the internal chiller of the Amicon SP 20. the concentrated lysate was then diafiltered against eight times its own volume (approximately 40L) of TE buffer until the conductivity was measured at ≤5 mS. The lysate was then further concentrated down to a volume of 3 L. Solid ammonium acetate was dissolved in the lysate solution with stirring to a final concentration of 2.5 M. The ammonium acetate solution containing the plasmid DNA was then incubated overnight at 4°C. The next morning, the resulting RNA precipitate was removed by centrifugation at 5000 rpm for 30 minutes in a Komposite 6 x 1 L rotor and Beckman J2-MI centrifuge. The supernatant containing the plasmid DNA was decanted and stored at 4°C prior to chromatography or Triton X-114 endotoxin removal.

TRITON X-114 ENDOTOXIN REMOVAL

Triton X-114 was added to the plasmid DNA supernatant (recovered after the ammonium acetate precipitation) for a final concentration of Triton X-114 of 2%. The mixture was stirred and incubated overnight at 4°C. The solution was then brought to room temperature the next morning and approximately 70% of the visible Triton X-114 phase was carefully pipetted off. The plasmid solution was then adjusted to a conductivity of between 38 mS - 42 mS by dilution with 50 mM Tris buffer, pH 7.5.

FRACTOGEL TMAE ANION EXCHANGE CHROMATOGRAPHY

TMAE fractogel anion exchange resin was packed into a Pharmacia XK 50 column, giving a bed height of approximately 20.5 cm and a total column volume of approximately 400 ml. A smaller column, a Pharmacia XK 26 with a total column volume of approximately 100 ml was also packed and used for two experiments in

which the effects of column capacity were further addressed. Prior to loading the column, all plasmid samples were adjusted to a conductivity of between 38 mS -42 mS and adjusted to a pH of 7.5 The column was run on a PerSeptive BioSystems BioCAD 250 preparative HPLC at a linear flow rate of 150 cm/hr. Chromatographic profiles were monitored at two wavelengths: 260 nm and 280 nm. All peak fractions were manually collected based on their real time chromatographic profiles.

The chromatographic protocol, optimized for the removal of genomic DNA and residual RNA, is briefly summarized as follows:

Column Equilibration:	50 mM Tris, pH 7.5, 500 mM NaCl
Sample Load:	Plasmid in 50 mM Tris, pH 7.5, 500 mM NaCl
Column Wash:	50 mN Tris, pH 7.5, 0.68 M NaCl
Stringent Wash:	**40 % Ethanol + 5 % Acetic Acid**
Column Elution:	50 mM Tris, pH 7.5, 1.2 M NaCl
Column Cleaning:	0.2 N NaOH + 1 M NaCl
Column Regeneration:	0.1 N HCl + 1 M NaCl

LIMULUS AMBOYCTE LYSATE ASSAY

The LAL kinetic assay (to assay for levels of endotoxin) was performed on individual plasmid fractions according to SOP 3-QC-0048

QUANTITATION AND ANALYSIS OF PLASMID DNA

Plasmid DNA was analyzed for total size and for the presence of residual genomic DNA and RNA by 1 % agarose gel electrophoresis using the Uni-Lane™ Gel System (0.5 x TBE 1 % GT-1) from Geno Technology, Inc., St. Louis, MO. The molecular weight marker was the 10 kb UniMarker™ from Geno Technology.

Plasmid DNA concentrations for individual fractions were determined at a wavelength of 260 nm using and extinction coefficient of 50 μg / ml.

MICRO BICINCHONINIC ACID (BCA) ASSAY

Residual protein content within the individual plasmid DNA fractions (post chromatography) were assayed using the Pierce Micro BCA kit.

α INTERFERON PLASMID TRANSFECTION EFFICIENCY

Transfection efficiency was determined by the CytoScreen™ Immunoassay Kit, BioSource International, Inc., Camarillo, CA., for quantitative detection of human Interferon-alpha. Cultures were seeded with approximately 1.0 x.10^6 HepG2 cells in a total volume of 4 ml in T25 cell culture flasks. The media used was DMEM supplemented with FBS and glutamine. Cultures were grown for 24 hrs and then transfected with 2.5 ng of plasmid DNA per ml of media using a calcium phosphate transfection method. Supernatents were collected from each flask and assayed for secreted α-interferon.

RESULTS and DISCUSSION

Intitally, as part of the present invention, the upstream processing steps of cell lysis, ultrafiltration and RNA precipitation were optimized to design an anion exchange elution profile that effectively removed residual genomic DNA, RNA and lowered the residual protein to undetectable levels (less than 0.5 μg/ml as measured by the Pierce Micro BCA kit). By removing a significant amount of the contaminating RNA by ammonium acetate precipitation, the Fractogel TMAE resin provided the resolution (as well as yields) necessary for a feasible large scale process.

Figure 2 shows a typical chromatogram produced from a large scale TMAE column purification while Figure 6 shows the corresponding 1 % Agarose gel results. Excellent separation and resolution between the contaminating species (genomic DNA, RNA and protein) and plasmid DNA is achieved. However, LAL analysis of plasmid preparations revealed high endotoxin levels.

Table 1 below lists a series of purification experiments, their key experimental parameters, and their final results. It was discovered as part of the invention that the anion exchange resin had a finite capacity for endotoxin clearance which is well below

its binding capacity for plasmid DNA. Even though column capacity experiments had shown no loss of resolution or yield at concentrations of 7.5 mg plasmid / ml resin, endotoxin levels at this loading concentration were unacceptably high. Small scale anion exchange purification can reduce endotoxin levels to acceptable levels but large columns, used at their maximum capacity, do not have sufficient endotoxin clearance. As shown in Table 2, the current industry specification for endotoxin in parenteral gene therapy DNA formulations is

< 0.1 EU / μg DNA (VICAL inc., Qiagen GmbH) .

TABLE 1: CHROMATOGRAPHY RESULTS

Expt. Condition	Total DNA	Resin volume (ml)	Load (mg /ml)	Total Endotoxin Load (EU)	EU / ml of resin	DNA Yield (mg)	EU/μg of DNA
Standard	3000	400	7.5	2.4×10^7	6.0×10^4	368	1.633
Standard	310	100	3.1	6.8×10^5	6.8×10^3	20.8	.046
Standard + Ethanol Wash	310	100	3.1	6.8×10^5	6.8×10^3	16.5	Unde-tectable
Standard + Ethanol Wash	1242	400	3.1	1.0×10^7	2.5×10^4	235	0.112
Standard + Triton	1242	400	3.1	1.0×10^7	2.5×10^4	191	0.016
Standard + Triton + Ethanol	1242	400	3.1	1.0×10^7	2.5×10^4	208	0.014

TABLE 2 - PLASMID SPECIFICATIONS

ASSAY	METHOD	SPECIFICATION
DNA homogeneity	Agarose gel	>90%CCC
	HPLC	
E. coli chromosomal	Slot blot hybridization	<1%
DNA		
RNA	Agarose gel	non-visualized
	Slot blot hybridization	
Endotoxin	LAL kinetic	<0.1 EU/ug pDNA
Identify	Restriction digestion	conforms to map
Sterility	USP membrane filtration	no colonies at 14 days
Purity	A_{260}/A_{280}	1.75 - 2.0
	A_{260}/A_{230}	>2.2
Protein	OD scan	$\lambda_{min} = 230$
	BCA micro titer	undetectable
Potency	transfection assay	"X" ng/ug reporter gene

When a stringent wash step (40% ethanol 5.0 acetic acid) was incorporated within the current chromatography protocol, a significant endotoxin reduction was seen. On a small scale column, endotoxin levels were below the limit of detection for the LAL kinetic assay while on a large column the endotoxin level was just slightly higher than the required specification. Compared to a purification without the stringent wash, a greater than 10 fold reduction in final endotoxin levels was achieved. While these

greater than 10 fold reduction in final endotoxin levels was achieved. While these experiments were carried out using endotoxin free buffers, they were not performed in a purification suite (class 1000 or better). The chromatographic profile (Figure 4) remains similar to the standard chromatographic method. It was, however, found to be necessary to lower the wash step following the ethanol + acetic acid wash to 0.5 M NaCl (rather than a continuation of the 0.68 M NaC1 wash step) in order not to lose product.

To streamline the large scale process and eliminate the need for subsequent centrifugation steps, a 2 % Triton X-114 phase partitioning step was used to remove endotoxin prior to anion exchange chromatography. Triton X-114, being a nonionic detergent, flows through the column and does not interfere with plasmid binding to the resin. The chromatographic profile and 1 % agarose gel form this method can be seen in Figure 3 and Figure 7, respectively. What is apparent is that there is some loss of product during the 0.68 M NaCl wash step (lane 8 on the agarose gel) and a slightly lower column yield (see table 1). What is not as easily quantified is the residual amount of Triton X-114 present in the final plasmid product. During the chromatography procedure, it was possible to visualize Triton in all the wash fractions (a phase separation could also be induced) and in the earliest eluting plasmid fraction, and it was observed that the presence of Triton X-114 interfered with the LAL kinetic assay.

Accordingly, as part of the invention, both the 2 % Triton X-114 pre-chromatography step and the stringent ethanol wash step were combined into the chromatography process. The results can be seen in Figure 5 and Figure 8. By including the ethanol wash step, the lowest endotoxin levels were achieved in the final plasmid product as well as no visible loss of product during the 0.68 M NaC1 wash step. Equally significant, the 40 % ethanol +5 % acetic acid wash step stripped off the residual Triton X-114. The most likely explanation for Triton X-114 being present is that it must remain associated with biomolecules bound to the column. The ethanol wash step (and subsequent salt wash) offers a major improvement over using just 2 % Triton as the sole endotoxin removal method.

To fully determine if this combined method for endotoxin removal during large scale plasmid production was feasible, the functional activity of the resulting plasmid product was investigated. Figure 9 compares in tabular form the transfection efficiency for the α-interferon plasmid produced by this large scale method versus a standard laboratory plasmid "maxi prep" kit. Figure 9 also graphically displays these results. Within the limitations of this biological essay, the plasmid produced by the large scale method functioned as well, if not better than, the Qiagen "maxi prep" kit sample. Assay variability was much lower for the transfections involving the large scale plasmid product.

Finally, to ensure that the ethanol + acetic acid wash condition did not lower the functional activity of the α - interferon plasmid, its transfection efficiency was compared to that of plasmid treated with Triton X-114 alone. The results are shown in tabular form in Figure 9. Again, within the variability of the assay, no significant activity differences could be seen between these two different treatments.

Conclusion

The experiments described above demonstrate that the use of a single anion exchange chromatography purification step can produce parenteral grade plasmid DNA. Having optimized the upstream unit operations of cell lysis, extraction and clarification, the present invention enables the incorporation of 2 % Triton X-114 phase partitioning prior to chromatography, as well as stringent wash steps (40% ethanol + 5 % acetic acid) during chromatography to maximize the utility of the TMAE Fractogel resin.

Most significantly, the use of this novel "mixed mode" chromatography wash step, which provides resolution based on differences in hydrophilicity between macro molecules rather than solely by ionic charge, greatly reduces endotoxin level while also providing the benefit of residual detergent (Triton X-114) removal. Used by itself, this wash step produces plasmid DNA of very high quality with undetectable endotoxin level. When used in conjugation with Triton X-114, this wash step produces a high capacity, fully scaleable process method for the production of parenteral grade plasmid

DNA. The plasmid DNA from this large scale process is capable of transfection efficiencies equal to laboratory standards.

Equivalents

Although the invention has been described with reference to its preferred embodiments, other embodiments can achieve the same results. Those skilled in the art will recognize or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific embodiments described herein. Such equivalents are considered to be within the scope of this invention and are encompassed by the following claims. All publications cited herein, including patents and pending patent applications, are hereby incorporated by reference in their entirety.

What is claimed is:

1. A method of purifying plasmid DNA comprising:

applying a cell lysate to a chromatographic column;

washing the column with a solution comprising a sufficient amount of an alcohol to substantially remove endotoxin bound to the column, without substantially removing plasmid DNA bound to the column;

eluting plasmid DNA bound to the column.

2. The method of claim 1, wherein the chromatographic column is an anion exchange column.

3. The method of claim 2, wherein the anion exchange column is a TMAE anion exchange column.

4. The method of claim 1, wherein said solution for washing said column comprises between 25% and 90% ethanol.

5. The method of claim 1, wherein said solution for washing said column comprises at least 40% ethanol.

6. The method of claim 5, wherein said solution further comprises at least 5% acetic acid.

7. The method of claim 1, further comprising adding a non-ionic detergent to the cell lysate prior to applying the cell lysate to the column.

8. The method of claim 7, wherein said detergent is Triton X-114.

9. The method of claim 7, further comprising removing the detergent phase formed after adding the non-ionic detergent to the cell lysate, prior to applying the cell lysate to the column.

10. The method of claim 1, further comprising collecting the eluted plasmid from the column.

11. The method of claim 1, further comprising washing the column with a salt solution prior to washing the column with the ethanol solution.

12. The method of claim 1, further comprising:

clarifying the cell lysate to remove large cellular debris; and

concentrating the cell lysate to remove RNA precipitate, prior to applying the lysate to the column.

13. A method of purifying plasmid DNA from a cell lysate comprising:

applying a cell lysate to an anion exchange column;

washing the column with a solution comprising a sufficient amount of ethanol to substantially remove endotoxin bound to the column, without substantially removing plasmid DNA bound to the column;

eluting plasmid DNA bound to the column.

14. The method of claim 13, further comprising adding a non-ionic detergent to the cell lysate prior to applying the lysate to the column.

15. The method of claim 14, wherein the detergent is Triton X-114.

16. The method of claim 13, wherein the solution for washing the column comprises at least 40% ethanol.

17. The method of claim 16, wherein said solution further comprises at least 5% acetic acid.

18. The method of claim 13, wherein the anion exchange column is a TMAE fractogel anion exchange column.

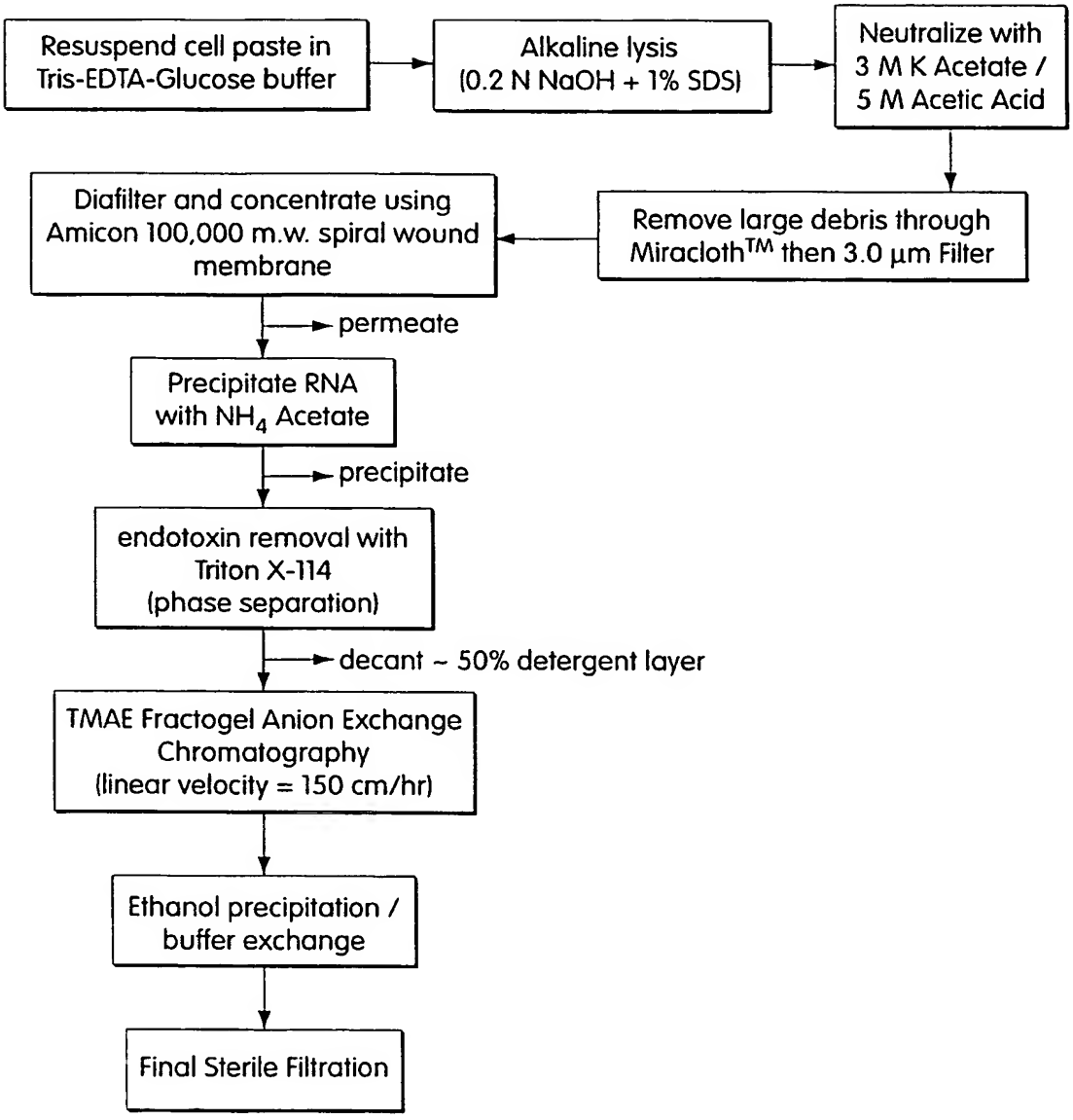

Resuspend cell paste in Tris-EDTA-Glucose buffer
Alkaline lysis (0.2 N NaOH + 1% SDS)
Neutralize with 3 M K Acetate / 5 M Acetic Acid
Remove large debris through Miracloth™ then 3.0 µm Filter
Diafilter and concentrate using Amicon 100,000 m.w. spiral wound membrane
permeate
Precipitate RNA with NH_4 Acetate
precipitate
endotoxin removal with Triton X-114 (phase separation)
decant ~ 50% detergent layer
TMAE Fractogel Anion Exchange Chromatography (linear velocity = 150 cm/hr)
Ethanol precipitation / buffer exchange
Final Sterile Filtration

Fig. 1

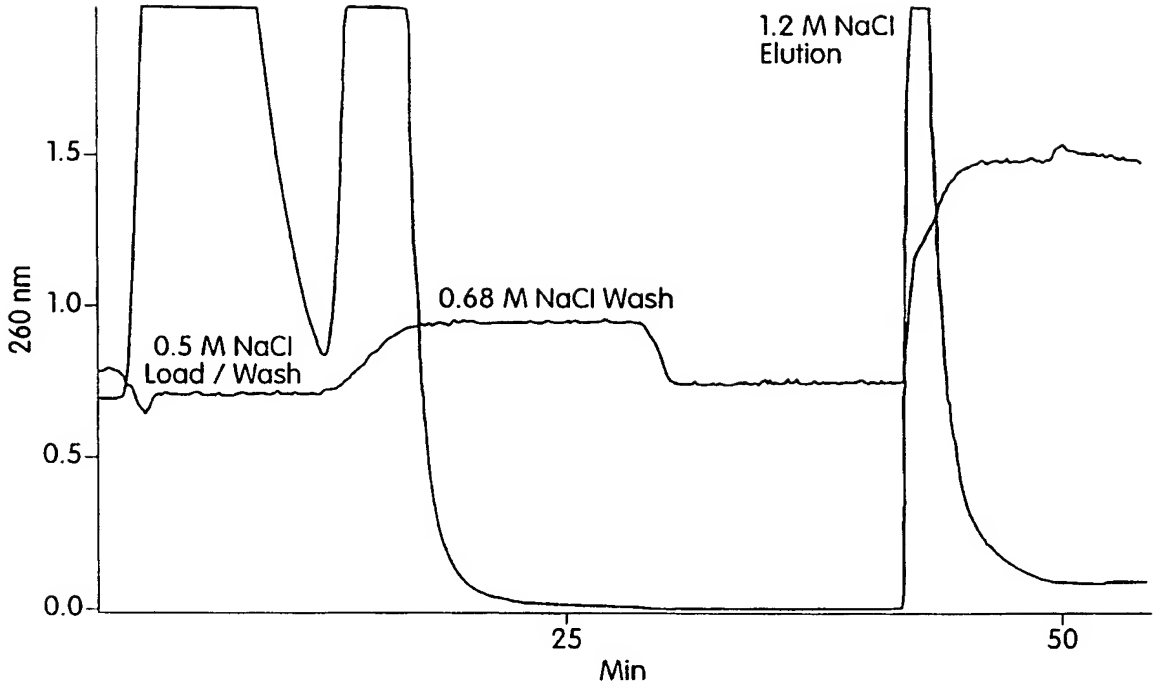

1.2 M NaCl
Elution
1.5
1.0
0.5
0.0
260 nm
0.68 M NaCl Wash
0.5 M NaCl
Load / Wash
25
50
Min

Fig. 2

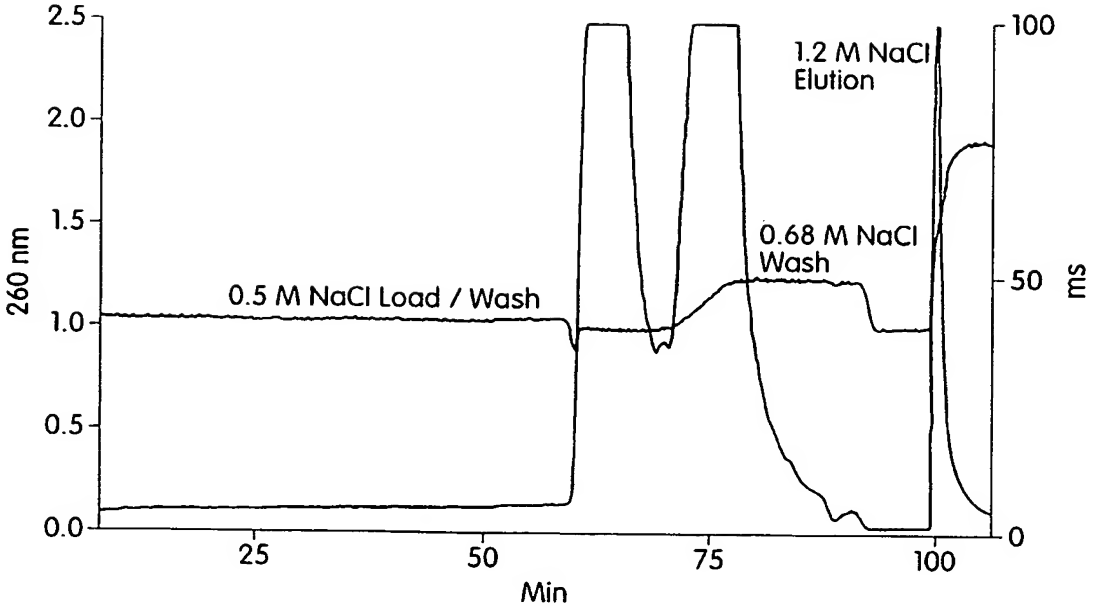

2.5
2.0
1.5
1.0
0.5
0.0
260 nm
0.5 M NaCl Load / Wash
1.2 M NaCl
Elution
0.68 M NaCl
Wash
100
50
0
ms
25
50
75
100
Min

Fig. 3

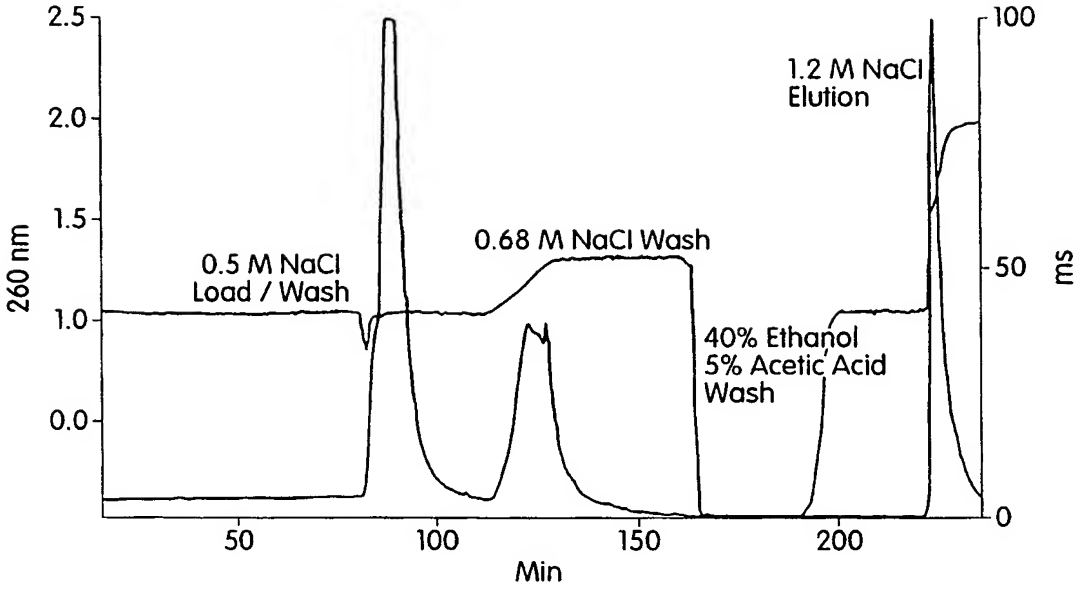

2.5
2.0
1.5
1.0
0.0
260 nm
100
50
0
ms
50
100
150
200
Min
0.5 M NaCl
Load / Wash
0.68 M NaCl Wash
40% Ethanol
5% Acetic Acid
Wash
1.2 M NaCl
Elution

Fig. 4

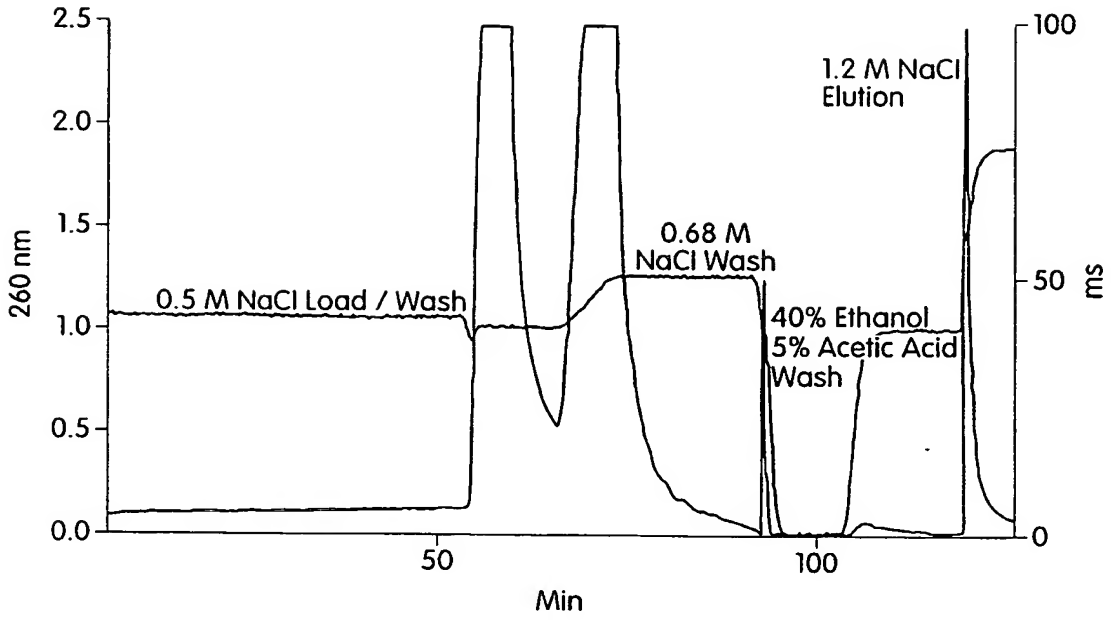

2.5
2.0
1.5
1.0
0.5
0.0
260 nm
0.5 M NaCl Load / Wash
0.68 M
NaCl Wash
40% Ethanol
5% Acetic Acid
Wash
1.2 M NaCl
Elution
100
50
0
ms
50
100
Min

Fig. 5

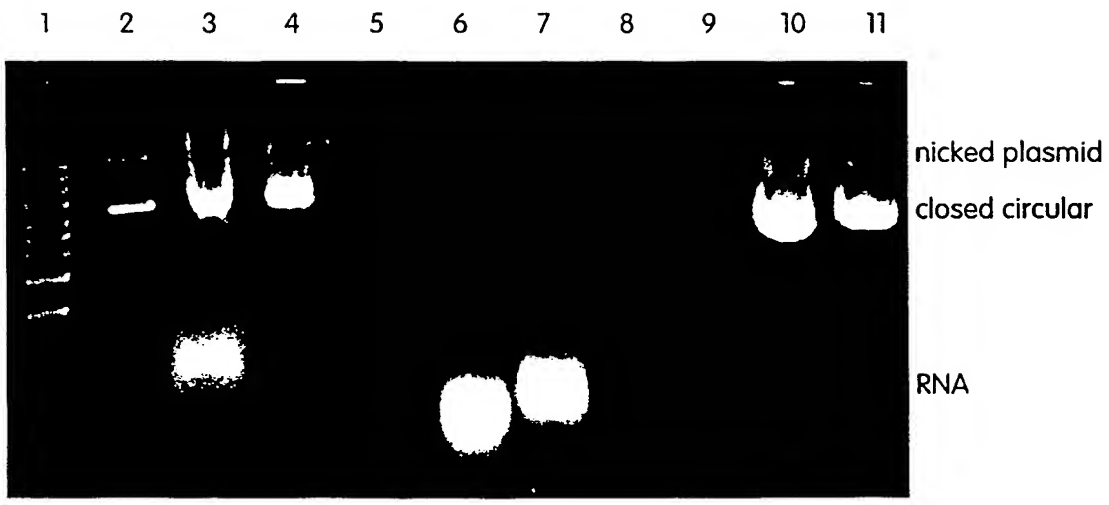


Fig. 6

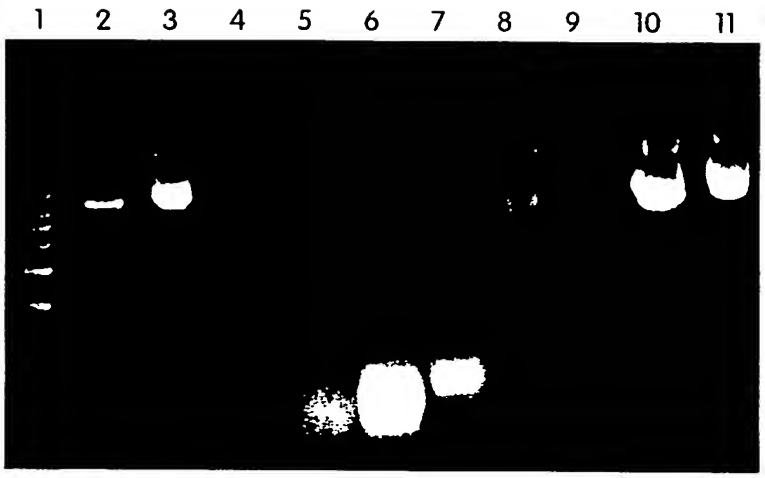


Fig. 7

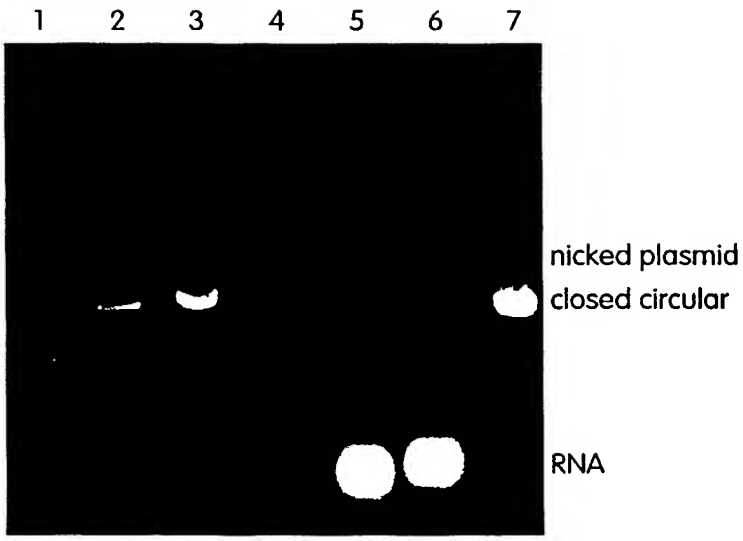


Fig. 8

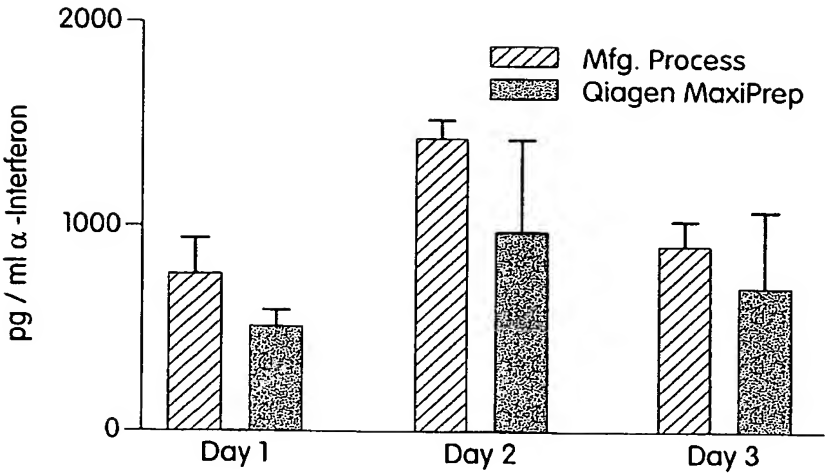

2000
1000
0
pg / ml α -Interferon
Mfg. Process
Qiagen MaxiPrep
Day 1
Day 2
Day 3

Fig. 9

INTERNATIONAL SEARCH REPORT

International Application No
PCT/ 99/11588

A. CLASSIFICATION OF SUBJECT MATTER
-IPC 6 C12N15/10 G01N30/02 B01D15/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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A	DE 36 44 346 A (SAEULENTECHNIK DR ING HERBERT) 21 May 1987 (1987-05-21) page 3, line 30-65 ---	1-3,18
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 October 1999	22/10/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Mateo Rosell, A.M.

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INTERNATIONAL SEARCH REPORT

Inter ional Application No
PC1/US 99/11588

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
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Information on patent family members

International Application No
PCT/US 99/11588

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